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DETECTION OF MOLECULAR AND PHENOTYPIC EFFECT OF CURCUMIN AND CRANBERRY ON SOME VIRULENCE GENES OF PROTEUS MIRABILIS INDUCED NEPHROLITHIASIS IN RATS

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ABSTRACT

This study was done in the college of medicine /Babylon university from March 2014 to April 2014. A total of 20 adult male albino rats randomly subdivided in to 4 groups containing 5 rats in each group as the following:

Group A: In this group, all rats administered 0.5 ml normal saline 0.9% through the catheter via the urethra into the urinary bladder.

Group B: In this group, all rats received 0.5 ml of (bacteria 10^9 /ml) through the catheter via the urethra into the urinary bladder.

Group C: All rats in this group administered 0.5 ml of (bacteria 10^9 /ml) through the catheter via the urethra into the urinary bladder and treated with curcumin in dose (100 mg\kg\day, p.o).

Group D: all rats in this group administered 0.5 ml of (bacteria 10^9 /ml) through the catheter via the urethra into the urinary bladder and treated with Cranberry (7mg/kg/day, orall).

Some virulence gene of *P.mirabilis* such as mrpA and ureC are investigates and it is found that this two genes (mrpA and ureC) is present in all isolates (group B)

The effect of curcumin and cranberry on urease were investigated, genetically there are no effect of curcumin and cranberry on the gene of urease while phenotypically it was stat the high concentration of it could cause inhibition to urease production

Also ,the effect of curcumin and cranberry on adhesion factor was also studied it was found genetically there are no effect of curcumin and cranberry on the gene of adherencegene while phenotypically that found failed to formation agglutination in the presence of D-mannose that treated with curcumin and cranberry.

These data show that curcumin and cranberry can provide protective effect against nephrolithiasis this protective effect of curcumin and cranberry may be related to the antioxidant status on the kidney.

KEYWORDS: *P.mirabilis*, mrpA, ureC, Curcumin, Cranberry

INTRODUCTION

Nephrolithiasis is a condition involving the formation of stone in the kidney; this is a common propagation disease with a percent of 5-8 globally (1).

P. mirabilis is best known for its ability to form stones in the bladder and kidney, as well as its ability to form crystalline biofilms on the outer surface and in the lumen of indwelling urinary catheters (2). Urolithiasis (stone formation), a major clinical problem in patients infected with *P. mirabilis*, is caused by the induction of urease, which hydrolyzes urea to ammonia, causing the local pH to rise and subsequent precipitation of magnesium ammonium phosphate (struvite) and calcium phosphate (apatite) crystals (3).

Adherence is a key virulence property of *P. mirabilis*. This organism attaches to and swarms across the surface of urinary catheters to gain a foothold in the urinary tract (4)

MrpA (the major structural subunit of the MR/P fimbriae), has a molecular mass of 18.5 kDa and is encoded by a 525-bp open reading frame (*mrpA*). MrpA was predicted to be a 175-amino-acid polypeptide with a 23-amino-acid hydrophobicleader sequence. It was found that MrpA was similar to uroepithelial cell adhesin these two proteins possess identical fragments of 10 amino acids) but not to the MR/P fimbriae isolated by (5) The MrpA is not absolutely required for infection, but contributes significantly to the ability of *P. mirabilis* to colonize the host. (6)

The most important *P. mirabilis* fimbriae seem to be MR/P(mannose resistant *Proteus* like fimbriae). This type of fimbriae is encoded by *mrp*operon containing 10 genes located on bacterial chromosome. Perhaps it is not surprising, then, that most of these proteins are also required for MR/P HA activity.Mannose-resistant, *Proteus*-like (MR/P) fimbriae, on the contrary, are expressed on the cell surface of *P. mirabilis* and should represent a candidate for the development of a vaccine to prevent *P. mirabilis* UTI and urolithiasis. The main structural subunit of these fimbriae is the MrpA protein (7). Many studies have suggested that MR/P fimbriae play a role in the virulence observed during UTIs caused by uropathogenic *P. Mirabilis* strains. Expression of MR/P fimbriae is increased under oxygen limitation. (6) were suggest that MR/P fimbriae dictate the localization of bacteria in the bladder.

Urease (urea amidohydrolase) is a nickel metalloenzyme which catalyzes the hydrolysis of urea into ammonia and carbamate. *Proteus mirabilis* is a well-known ureolytic human’s pathogen. Urease is one of the major bacterial virulence factors during urinary tract infections caused by these bacteria. Urease is very important in *P. mirabilis* pathogenesis. This enzyme catalyze the formation of kidney and bladder stones or to encrust or obstruct indwelling urinary(8). The urease gene cluster of *P. mirabilis* encodes three structural polypeptides, UreA, UreB, and UreC, which form the apoenzyme; four accessory polypeptides, UreD, UreE, UreF, and UreG; and an AraC-like positive transcriptional activator, UreR. The urea-inducible urease gene cluster (ureRDABCEFG) encodes a multimeric nickel-metalloenzyme that hydrolysing urea to ammonia and carbon dioxide, thereby increasing the pH and facilitating the precipitation of polyvalent ions in urine (stone formation) (9). This pH alteration is important during *P. mirabilis* catheter colonization, facilitating the bacterial adherence and formation of biofilm incrustation. The gene ureC, which encodes the large subunit of the enzyme, was used as a target for gene disruption, rendering the bacterium unable to synthesize a functional urease. Stone formation is the primary role of urease during UTI caused by urease-producing organisms. A secondary role of urease during *Proteus* UTI is the accumulation of toxic levels of ammonia from urease-mediated hydrolysis of urea that damages tissue including renal epithelia. Stone formation is a hallmark of P. mirabilis infection, supplying a number of advantage including, the host

immune system protection, blockage of the ureters, ammonia toxicity to host cells, and direct tissue damage. (10) determined that urease of *P. mirabilis* is a critical virulence determinant necessary for colonization, urolithiasis, and severe acute pyelonephritis.

The cranberry was primarily used as a traditional medicine for the treatment of bladder and kidney ailments among American Indians (11). On the other hand, curcumin is widely studied and it is found that curcumin possesses also antibacterial (12), antifungal (13) and antiviral (14) properties. These studies suggest that curcumin is a potent agent, which maybe applied in various pharmacological areas. Additionally, curcumin does not exhibit toxicity to either animal or humans even at high doses.

MATERIALS AND METHODS

Experimental Animals

For Nephrolithiasis induced by *Proteus mirabilis*20 adult male albino rats weighting (190-280 g) for

The animal were purchased from the college of Veterinary medicine/ University of Baghdad. They were housed in the animal house of Babylon university/ College of Medicine(for two weeks) in a temperature-controlled (25°±1C) room (humidity was kept at 60–65%) with alternating 12-h light/12-h dark cycles and were allowed free access to water and chow diet until the start of experiments tables.

The experimental period were from March 2014 to April 2014 done in the animal house of Babel University college of medicine.

A total of 20 adult male albino rats weighting (190-280 g) Randomly Subdivided in to 4 groups containing 5 rats in each group as the following:-

Group A (Control group) in this group, all rats received regular rat food and drinking tap water and administered 0.5 ml normal saline 0.9% through the catheter via the urethra into the urinary bladder.

Group B (Induced group) served as nephrolithiasis induced group by administered 0.5 ml of (10^9 bacteria /ml) through the catheter via the urethra into the urinary bladder.

Group C (Curcumin group) all rats in this group administered 0.5 ml of (10^9 bacteria/ml) through the catheter via the urethra into the urinary bladder and treated with Curcumin in dose (100 mg\kg\day, p.o) (Shukla et al., 2008).

Group D (Cranberry group) all rats in this group administered 0.5 ml of (10^9 bacteria /ml) through the catheter via the urethra into the urinary bladder and treated with Cranberry (7mg/kg/day, orally)) (Cheshchevik et al., 2012).

DNA Extraction for Gram Negative Bacteria

This method was performed according to the genomic DNA purification kit supplemented by a manufacturing company (Geneaid/Uk)

Detection of Some Virulence Genes by PCR

DNA (extract from bacterial cells) was used as a template in specific PCRs for the detection of some of *Proteus miriblis* virulence genes. DNA was purified from bacterial cells by using the Geneaid DNA extraction Kit. The primers used for the amplification of a fragment gene were listed in Table 1:

Table 1: Primers Sequences and PCR Condition

Genes	Primer Sequence (5′-3′)	Size of Product bp	PCR Condition	Reference
ureC F ***ureC R***	CCGGAACAGAAGTTGTCGCTGGA GGGCTCTCCTACCGACTTGATC	533	***94 ºC 3min 1x*** 94 ºC 1 min 63 ºC 30sec 30x 72 ºC 1 min 72ºC 7 min 1x	(15)
mrpA ***F*** mrpA **R**	GAGCCATTCAATTAGGAATAATC CA AGCTCTGTACTTCCTTGTACAGA	**648**	95 ºC 5 min 1x 94 ºC 1 min 58 ºC 1 min 35x 72 ºC 1 min 72ºC 7 min 1x	(16)

Mannose-Resistant Haemagglutination (MRHA)

For this method, the bacteria were grown on blood agar plates. After overnight incubation at 37^{o}C, 1 to 2 single colonies were picked and suspended in saline (0.85% NaCl) to give a turbid suspension. Red blood cells suspension (3%) in phosphate buffer saline (PBS) was prepared by washing fresh citrated blood group A^{+} (2000 x g, 10min). The test was performed in the presence of 0.5% (w/v) D-mannose according to (17).

On a clean slide , one drop of bacterial suspention is mixed with one drop of D-mannose on one slide , one drop of blood group A with one drop of 3% suspention on the other slide (with and without of 1% of curcumin& cranberry) The agglutination of RBC with bacteria is detected after 0.5 to 2 min. in room temp. (special communication)

Effect of Cranberry and Curcummin on Urease Assay

Overnight culture of the strain was diluted 1:1000 in LB containing ampicillin (50 mg/mL) and cranberry and curcummin at 0, 1, 5, 10, and 15 mg/mL and was incubated for 3 h. The bacteria were then induced with urea and harvested after 3 h of additional growth. Induced bacteria were washed twice in phosphate-buffered saline (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4, and 0.24 g of KH2PO4 per litre (pH 7.4)), , the absorbance is read at wave length OD600 by (18).

RESULTS

Molecular Detection of UreC Gene

Molecular detection of urease gene was done by using specific PCR primer. It was found that ureC marker was observed in all *P. mirabilis* isolates (group B) with molecular size (533bp) as shown in figure 1.

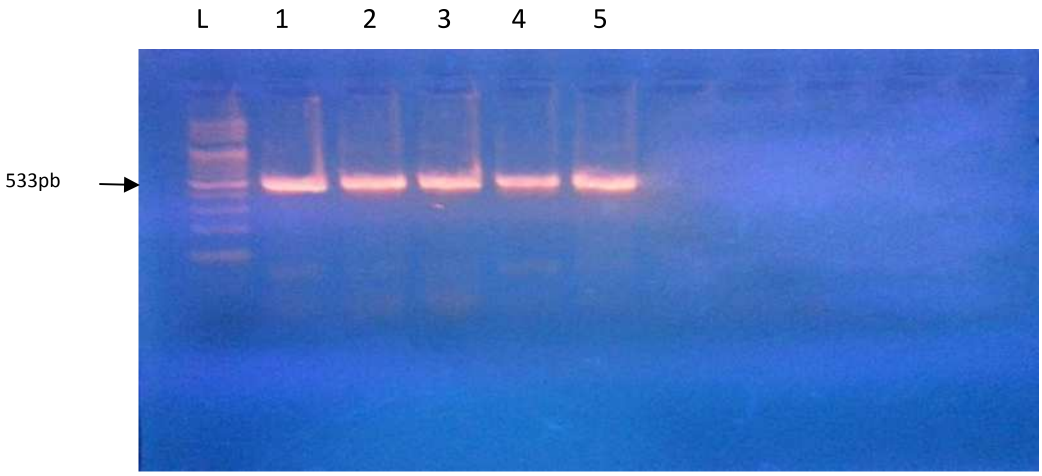

Figure 1: Gel Electrophoresis of PCR of *UreC* amplicon Product. L: Ladder; 1-5: No. of Isolates Obtained from Urine of Rate

Effect of Cranberry and Curcummin on Urease

In this study, it was shown that cranberry and curcummin cannot effect on the urease gene (group C & D)as shown in figure 2

On the other hand, the effect of Cranberries and curcumin at different concentration (0, 1, 5, 10, 15 µg/ml) on urease of *Proteus mirabilis*(group C & D) have been investigated as shown in figure 3 & 4

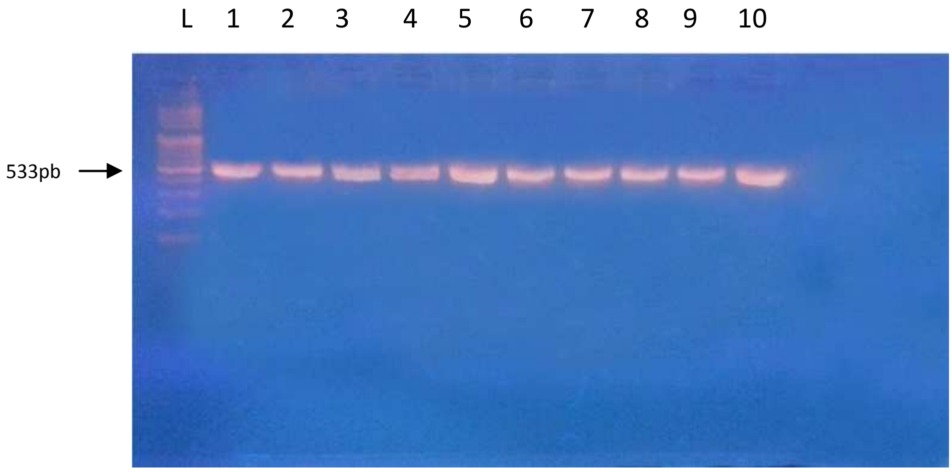

Figure 2: Gel Electrophoresis of PCR of *UreC* amplicon Product. L: Ladder; 1-5: No. of Isolates Obtained From Urine of Rate After Treatment with Cranberry, 6-10: No. Of Isolates Obtained From Urine of Rate after Treatment with Curcumin

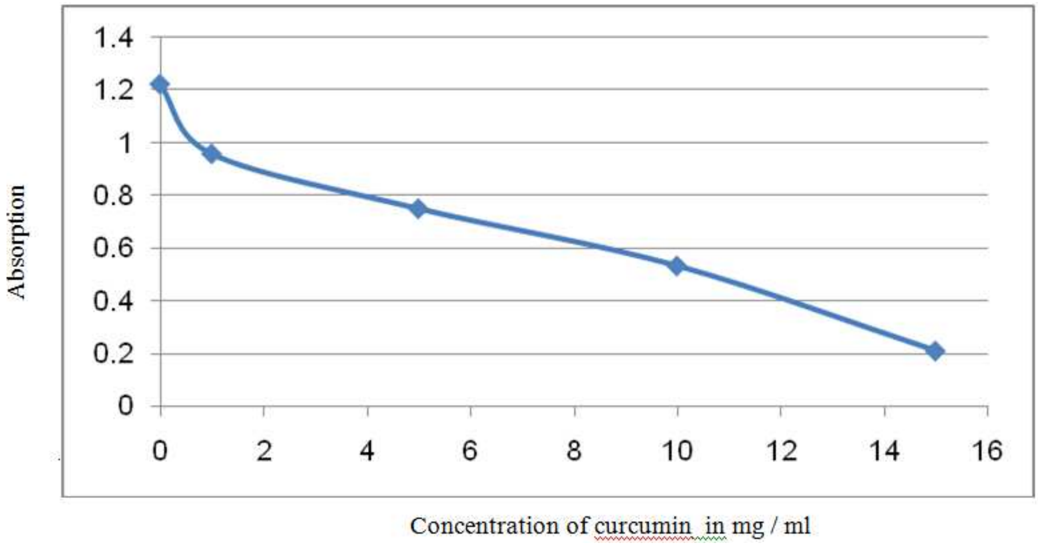

Figure 3: The Effect of Curcumin on the Urease of *P. mirabilis*

Table 1: Effect of Curcumin on the Urease of *P. mirabilis*

Concentration of Curcumin	Absorption
0	1.222
1	0.958
5	0.750
10	0.533
15	0.211

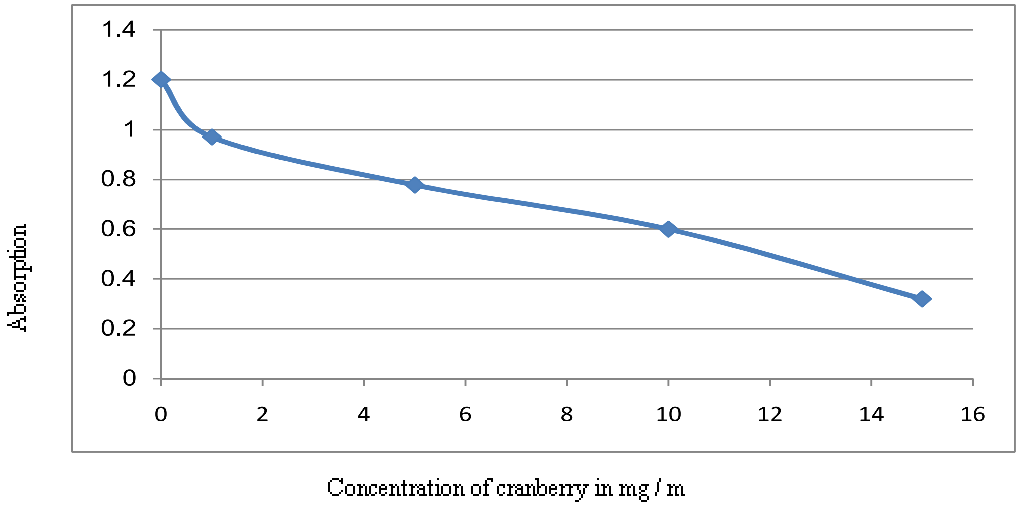

Figure 4: the Effect of Cranberry on the Urease of *p. mirabilis*

Table 2: Effect of Cranberry on the Urease of *p. mirabilis*

Concentration of Cranberry in mg	Absorption
0	1.200
1	0.970
5	0.777
10	0.600
15	0.320

Detection of Adhesion in *P. mirabilis*

mrpA gene was detected in *P. mirabilis* isolates by using specific primer, it was found that all isolates (group B)give positive amplicon for this gene as shown in figure 5

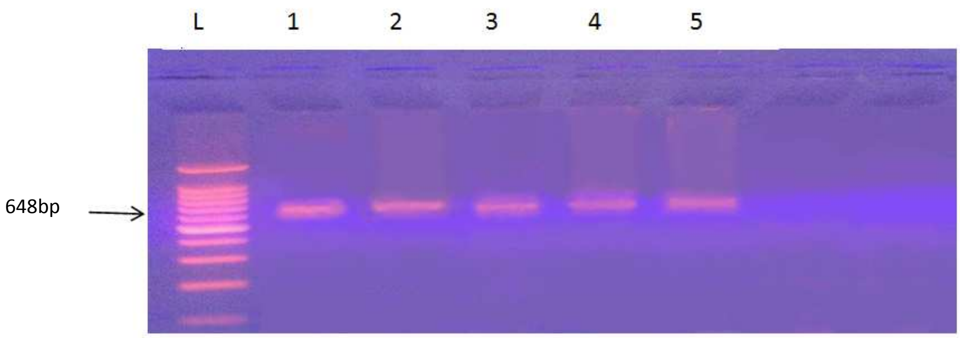

Figure 5: Gel Electrophoresis of PCR of *Mrpa* amplicon Product.
L: Ladder; 1-5: No. of Isolates Obtained From Urine of Rate

Effect of Cranberry and Curcumin on Adhesion Factors

In this study , genetically, it was shown that Cranberries and curcumincan not effect on adhesion gene (group C & D) as shown in figure 6, while results shown that Cranberries and curcumin can effect phenotypic expression of *p fimbriae* by faild to formation of aggregation(group C & D) as shown in figure 7.

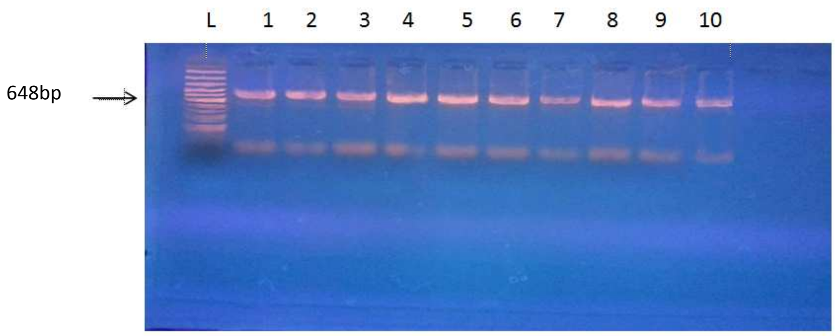

Figure 6: Gel Electrophoresis of PCR of *Mrpa*amplicon Product.
L: Ladder; 1-5: No. of Isolates Obtained From Urine of Rate After Treatment with Cranberry, 6-10: No. of Isolates Obtained from Urine of Rate after Treatment with Curcumin

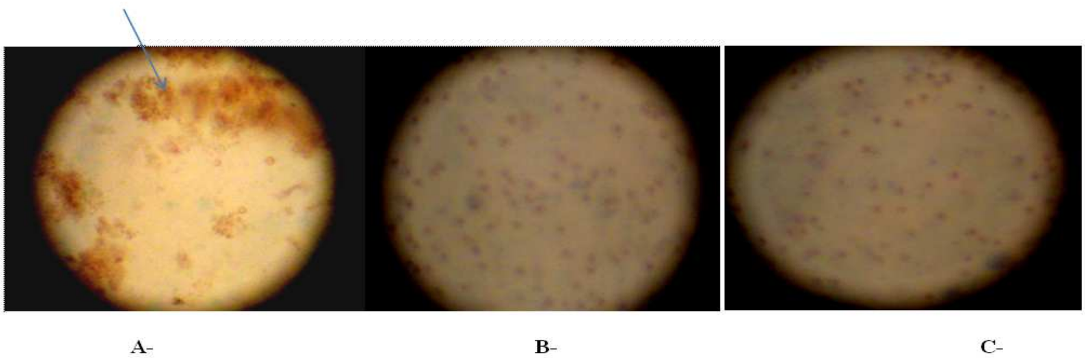

Figure 7: Effect of Crucumin and Cranberry on Adherence Factors (40X)
A: Control, B:-Curcumin, C:- Cranberry, → Indicate of Aggregation of Bacteria

DISCUSSIONS

Detection of Adhesion in *P. mirabilis*

Adhesion is considered primary factor which cause adhesion of bacteria to the target cell of the host, and their presence indicates that the bacteria contain cell surface fimbrial antigens. This results is identical to results obtained by (19) who found that *P. mirabilis* mrpA fimbrial genes is present in all isolates expressed mrpA (MR/P). This particular type of fimbriae are frequently related to *P. mirabilis* UTI pathogenesis.

On the other hand, (20) we found that 69 of 71 isolates screened genotypically for *mrp* genes or phenotypically for mannose-resistant hem agglutination of erythrocytes, were positive (*mrp* or MR/P).

phenotypic expression of p fimbriae can be detected by mannose resistant haemagglutination of human erythrocyte. The result of this study shows the same results of mrpA, indicating positive correlation between the level of MR/P and the number of mrpA for the isolates.

Also, the presence of the MR/P-related genes mrpA was detected by PCR in all isolates. This results is identical to results obtained by (16) who found that this factor is present in all isolates.

Haemagglutination, a property that reveals specific adhesion receptor interactions, is associated with expression of P. mirabilis MR/P fimbriae (21) (22). When haemagglutination was evaluated within our isolate collection, it was observed that every tested isolate was able to agglutinate fresh human erythrocytes. As expected, the reaction was not inhibited by mannose P. mirabilis represents a particular case in which various types of fimbriae can be expressed simultaneously by the same cell (23). Several authors suggest that P. mirabilis fimbriae are implicated in the colonization of the urinary tract (7)(24).

Effect of Cranberry and Curcumin on Adhesion Factors

Cranberries and curcumin may not effect on gene but the effect may becauses temporary genomic silencing as aresult increasing in the epigenetic tool and hence the block protein synthesis or these can effect by suppressing of gene expression. On the other hand, Cranberriesand curcumin can effect phenotypic expression of *p fimbriae* (can be detected by mannose resistant haemagglutination of human erythrocyte) by faild to formation of aggregation . So there was no previous studies have indicate the effect of curcummin on adherence factor cranbary exert anti-adhesive effects on certain uropathogens (25) and this effect is specific to certain components of cranberry (26). Cranberries contain three different flavonoids (flavonols, anthocyanins and PAC), catechins, hydroxycinnamic and other phenolic acids and triterpenoids. The anthocyanins are absorbed in the human circulatory system and transported without any chemical change to the urine (27). Cranberry products do not inhibit bacterial growth, but inhibit bacterial adherence to uroepithelial cells, thereby reducing the development of UTI. The consumption of cranberry juice can help to prevent the adhesion of bacteria to the uroepithelium and thereby help reduce the incidence of UTIs. With rising concerns of antibiotic resistance among bacteria, cranberry could serve as an effective alternative in controlling UTIs. (28) investigated the effect of cranberry capsules on bacterial adherence to urinary tract.

Participants who consumed cranberry capsules showed a significant dose dependent reduction in bacterial adherence to urinary epithelial cells compared to placebo. When bacteria was grown in the presence of cranberry components, the bacterial morphology changed to a more spherical cell-like form. These changes cause them to be repelled by the human cells (29).

Molecular Detection of UreC Gene

Urease, a nickel metalloenzyme that catalyses the hydrolysis of urea, resulting in an elevation of pH, contributes to the formation of bladder and kidney stones in P. mirabilis UTI (3).The results is agreement to result obtained by (30) who found that this gene is present in *p. mirabilis* isolates.

urease can be associated with stone formation and serious kidney damage, it cannot be used as an index of virulence, since nearly all *P. mirabilis* strains produce this enzyme (20).

The genes encoding the enzyme are induced by substrate urea and are, thus, probably always being expressed during an active infection. There does not appear to be a feedback mechanism (such as high concentrations of ammonia) for shutting off synthesis of the enzyme. Therefore, there could be a relentless production of the enzyme in response to the constant elimination of urea as a nitrogenous waste by the host.

A strong association was noted between urease production and stone formation. Urolithiasis was observed only in animals infected with the parent strain, confirming that the mechanism of urinary stone formation, which is a feature of *P. mirabilis* infections (31) , is a result of urease-catalyzed hydrolysis of urea. Previous studies have demonstrated that urease is critical for crystallization of struvite stones(32). The process of urolithiasis appeared to require more than 2 days to produce visible stones since none of the infected animals was found with a stone after 2 days, whereas 31% (12 of 39) of the animals harbored stones after 1 week.

Early studies using *P. mirabilis* treated with urease inhibitors established a link between urease and colonization of rat urinary tracts. In these studies, the renal tissue of control-infected rats contained a far higher number of bacteria and had greater tissue damage than did those of rats infected with inhibitor-treated *P. mirabilis* (33).

Bacterial urease is recognized as a virulence factor because of its role in kidney and bladder stone formation. genetic analyses of several ureolytic bacterial species revealed that it is possible to eliminate urease activity by disrupting genes encoding proteins other than the urease subunits.

Urease activity raises pH of human urine, which allows precipitation of normally soluble polyvalent ions to struvite and carbonate apatite. These compounds aggregate around bacteria, forming urinary stones. Inside such stones, microorganisms are protected from antibiotics and the host’s immune system. Urinary stones block urethra or catheters leading to acute bacteriuria(32).

Effect of Cranberry and Curcummin on Urease

Cranberry and curcummin cannot effect on the urease gene but the effect may be can influence on the intra-locus between basis that make sequestration to the bases which give rise to make failing in transcription and protein synthesis.

It has been observed that increase cranberry concentration result in decreased urease expression. So this results is identical to result obtained by (34) have been found effect of cranberry powder (35) on urease expression.

On the other hand, the results were demonstrate that increase curcumin concentration lead to inhibits the activity of urease-bacterially produced enzyme, this results is similar to the results obtained by (36) have demonstrate that curcumin exhibits the effect against *Proteus mirabilis* inhibiting the activity of urease—an enzyme produced by these microorganisms. The results shown curcumin the is more effective on urease than cranberry.

Also, the results of this study is compatible to results obtained by (34) who have found that the increasing concentrations of cranberry powder reduce the production of urease by the bacteria, also foundcranberries prevent UTIs by hindering bacteria from sticking to the walls of theurinary tract. Urease inhibitors interact with it and block its activity by inhibiting the urea hydrolysis to ammonia and carbon dioxide. Urease inhibitors can be divided into two major categories: (i) active-site directed mode; (ii) mechanism-based directed mode. The active site directed inhibitors show close structural similarity with urea—the enzyme's substrate. Mechanism-based directed inhibitors interfere with the enzyme's catalysis mechanism leading to its inhibition or inactivation (37)

CONCLUSIONS

The genes of urease and adherences is present in all isolates (group B).Cranberry and curcumin cannot effect on urease gene genetically, but can effect phenotypically by decrease of urease production. On the other hand, Cranberry and curcumin cannot effect on adhesion gene genetically, but can effect phenotypically failed to form aggregation in the presence of D-mannose

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